Proteomic Analysis of Malignant Ovarian Cancer Effusions as a Tool for Biologic and Prognostic Profiling

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Abstract

Purpose: Malignant epithelial ovarian cancer effusions are important in disease dissemination and clinical outcome. The identification of biochemical events active in effusions may improve our identification and application of targeted therapeutics.

Experimental Design: Archival effusion samples for which outcome information was known were studied. Clinical variables were comparable between these groups. Two cohorts of patients with malignant effusion were assessed: those with effusion at presentation (Tap1) or at first recurrence (Tap2). Expression and activated fraction of selected signaling proteins were quantitated on serial protein microarrays using validated antibodies. Proteomic results and clinical variables were analyzed by univariate analysis followed by Cox proportional hazards model analysis.

Results: Malignant effusions (>80% malignant cells) were distinguished from benign effusions by higher expression of AKT, activated extracellular signal-regulated kinase, activated ($P \leq 0.001$) and total cAMP-responsive element binding protein ($P = 0.01$), and JNK ($P = 0.03$). Malignant pleural effusions could not be differentiated from ascites by signaling profiles. Both had signal expression clusters for survival, proliferation and metastasis, and injury pathways. Cox proportional hazards model analysis revealed high p38 and pEGFR/EGFR ratio as jointly associated with poor survival in Tap1 cases (both $P \leq 0.002$). Phospho-JNK quantity was associated with worse outcome in Tap2 patients ($P = 0.004$), when taking other factors into consideration.

Conclusions: Proliferation, survival, and apoptosis signaling dysregulation can be identified in ovarian cancer effusion samples. Biochemical characterization of clinical effusions may provide either predictive and/or correlative information on patient outcome from which to further understand the mechanisms of effusion development and target clinical intervention.

Ovarian cancer is the leading cause of death from gynecologic cancer in industrialized countries (1). Despite new chemotherapy regimens, only modest gains have been made in long-term patient survival. Patient mortality from ovarian cancer is largely attributed to widespread metastasis to the serosal surfaces and associated peritoneal and/or pleural effusions (2–4). Accumulation of effusions is the combined result of lymphatic obstruction, activation of native mesothelial cells by the malignant metastatic process, and increased vascular permeability driven by production and secretion of factors such as vascular endothelial growth factor and interleukins 6 and 8 (5–9). Advances in understanding the mechanisms underlying development and propagation of effusions will lead to improved therapeutic approaches.

Malignant epithelial cells and activated mesothelial cells are the primary components of malignant effusions. Activation of mesothelial cells could cause them to become phenotypically and functionally similar to the malignant cells (10). Both can produce cytokines, growth factors, and invasion-promoting components, further promoting the permissive local microenvironment (11). This rich medium provides support for malignant cells to proliferate and further metastasize despite the lack of matrix substrata, allowing these cells to overcome the apoptosis associated with loss of attachment. This implies that malignant cells and mesothelial cells have up-regulated survival signaling in order to survive in the hypoxic but otherwise rich liquid milieu. Identification of the critical signaling differences that promote and support cellular survival in effusions will allow targeting of this clinically problematic and poor prognostic process, thus improving patient comfort and outcome.

Development and validation of protein lysate microarrays has facilitated study of signaling pathways in ovarian carcinoma (12) and other cancers (13). Up-regulation of the extracellular

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signal-regulated kinase (ERK) mitogen-activated protein kinase proliferation pathway and of the AKT/phosphatidylinositol 3′ kinase pro-survival and invasion pathways has been shown in ovarian cancer (12, 14, 15). We hypothesized that application of this novel and very sensitive protein pathway profiling technology to viably frozen malignant and benign pleural and peritoneal effusions would provide biochemical insight into signaling events in malignant effusions. This is the first report to profile biochemical pathways active in malignant effusion samples. Our results have (*a*) identified signaling molecules that differentiate malignant from benign effusions, (*b*) documented correlation of signaling molecule expression and activation with clinical variables, and (*c*) has shown that activation of survival signaling pathways may be associated with survival end points, suggesting that they are logical molecular targets for investigation into their potential for therapeutic intervention.

Materials and Methods

Specimens and patient data. Specimens and relevant clinical data were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital under informed consent according to national Norwegian and institutional guidelines. The material consisted of 61 fresh malignant peritoneal and pleural effusions submitted between May 1998 and June 2003. Reactive (benign, 9), and malignant peritoneal (46) and pleural (15) effusions were obtained from patients with ovarian carcinoma (53), carcinoma of the fallopian tube (3), and primary peritoneal carcinoma (5). The tumor samples were not subjected to culture and were viably frozen to minimize bias due to processing. A small number of patients had multiple specimens from which only results from the first effusion in temporal sequence were used for clinical analysis. Specimens were processed within minutes of removal from the patient, suspended and frozen in equal volumes of RPMI medium supplemented with 20% FCS and 20% DMSO. Smears and cell block sections from formalin-fixed paraffin-embedded pellets underwent diagnostic evaluation and were further characterized using immunocytochemistry (Supplemental Fig. S1; ref. 16). Effusions were obtained from 25 patients for whom the effusions were present prior to initial therapeutic intervention (Tap1) and from the remaining 29 patients for whom the effusions developed and were sampled at first disease recurrence (Tap2); all but one of these patients received treatment with paclitaxel + platinum or single-agent platinum. Patients in these two groups had comparable clinicopathologic variables and were treated similarly (Supplemental Table A).

Immunocytochemistry. Immunocytochemistry was done as reported previously (16). Briefly, serial 3 to 4 μm sections from paraffin-embedded cellblocks were dried and dewaxed; the endogenous peroxidase quenched was then subjected to heat-induced epitope retrieval. Slides were then incubated with the primary antibody diluted in 1% bovine serum albumin for 2 hours at room temperature. Signal was developed using Envision+/horseradish peroxidase in 0.04% aminoethylcarbazole (Sigma, St. Louis, MO) in 0.05 mol/L acetate buffer (pH 5.0). Finally, slides were counterstained with Mayer's hematoxylin and coverslipped.

Lysate arrays and immunostaining. Arrays were constructed and protein expression was analyzed as described previously (17). Briefly, slides were incubated with I-block solution (Applied Biosystems, Foster City, CA) and immunostained on an autostainer according to the manufacturer's instructions (CSA kit, DakoCytomation, Carpinteria, CA). A single band specificity has been shown previously for the polyclonal primary antibodies applied (Supplemental Table B, http://home.ccr.cancer.gov/ncifdaproteomics/pmicroarray.asp). The negative control slide was incubated with antibody diluent and no primary antibody. The amount of total protein per microarray spot was determined with a Sypro Ruby protein stain (Molecular Probes, Eugene, OR) and imaged with a CCD camera (Alpha Innotech, San Leandro, CA). Each array was scanned, spot intensity integrated over a fixed area as described (18), and analyzed, data normalized to total protein, and a standardized, single data value was generated for each sample on the array. This single data point was used for comparison (ImageQuant Ver. 5.2, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Comparisons of expression levels between malignant and benign specimens, and between pleural and peritoneal effusions were done using the Wilcoxon rank sum test. Comparisons of expression levels between pairs of biochemical variables were made by dividing the expression level of each at its median and using the χ^2 test, with unadjusted two-tailed P values, to evaluate the statistical significance of the association in each of the resulting 2×2 tables. Data from the two subsets of patients for whom clinical outcome measures were known were analyzed: those with effusions at the time of diagnosis (Tap1; $n = 25$), and those for whom effusions developed at first clinical recurrence after receiving chemotherapy (Tap2; $n = 29$). Survival data was not obtained for one patient in Tap2, leaving 28 subjects for analysis. The two groups were analyzed separately because only the Tap1 patients' effusions potentially could be used in a predictive manner. Effusions in Tap2 would need to be considered correlates of outcome because they were obtained at progression and their protein profiles could have been influenced by the chemotherapy received and the clinical picture at recurrence. Survival was calculated from the date of diagnosis until date of death or date of last follow-up. The probability of survival was calculated using the Kaplan-Meier method, and the significance of the difference among survival curves evaluated was determined by the Mantel-Haenszel procedure (19, 20).

Variables evaluated for association with survival included whether the patient was never disease-free (recurrence at ≤1 month from completion of therapy), cell composition (% tumor cells), age, ascites versus pleural effusion, grade, residual disease, and Federation Internationale des Gynaecologistes et Obstetristes (FIGO) stage, in addition to the tested total of 39 total and phospho-proteins and phospho-to-total protein ratios. The 39 total and phospho-proteins, ratios, and other potential prognostic factors were evaluated individually in univariate analyses with exploratory intent. Given the very large number of variables being evaluated and the relatively small number of patients considered in each of the two groups, the resulting P values from the global log rank test for each variable were used to screen variables for subsequent evaluation in a Cox proportional hazards model (21). Expression in the first obtained specimen only was entered in survival analyses for patients with more than one effusion.

Age and the 39 protein level variables (40 total variables), were each initially divided into approximate quartiles of the observed variable distribution in order to identify if a potential difference in survival could be identified. The data, for those variables in which such a potentially useful prognostic classification could be identified, were regrouped into the two resulting categories and reevaluated for prognostic significance. In a definitive analysis, all P values resulting from this regrouped analysis would have needed to be adjusted for this implicit set of three evaluations. However, because this is an exploratory analysis, P values have been presented without any formal adjustment for multiple comparisons in order to simplify presentation of these results of univariate evaluations. The variables which would have required adjustment if interpreting the results more formally, are noted in the tables. Variables with an unadjusted $P < 0.15$ in the univariate analysis were considered for inclusion in subsequent Cox model evaluations. The data were dichotomized at the cut-point which had yielded the most significant result for the univariate analyses for the Cox models because the trends in overall survival were not necessarily linear over the four quartiles. As a result, the Cox model P values might be biased because the cut-points were selected based on an examination of the same data that were used in the model. The resulting model variables were converted to hazard ratios (HR) with associated 95% confidence intervals (CI; ref. 22). All P values are two-sided.

Results

Protein profile segregation of benign from malignant effusions. The diagnosis of malignant cells in effusions has major clinical prognostic significance for management in the primary setting and in disease recurrence. Several immunocytochemical markers have been shown to be useful in this setting. Effusion lysate aliquots were characterized using immunocytochemistry (16) with calretinin, a mesothelial marker (23), and CD45, a leukocyte marker, for cross-validation of the markers with the high-throughput protein array approach (Supplemental Fig. S1). Strong membrane staining was seen in tumor cells stained with Ber-EP4, with nuclear and cytoplasmic calretinin staining in the mesothelial cells. Quantitative results were obtained using lysate arrays for calretinin and CD45. These results agreed with the morphology and immunohistochemistry classification. CD45 (P = 0.004), calretinin (P = 0.03), and expression of both CD45 and calretinin (P = 0.008) were all lower in specimens that consisted predominantly (80-100%) of malignant cells compared with malignant effusions containing larger populations of reactive cells and benign effusions. This analysis shows that lysate array proteomics might be used as a tool to discriminate benign from malignant effusions. Effusions of 80% or greater malignant cells were selected for further study in order to enrich for signals from the malignant component of the effusions.

Little is known about the signaling events in activated mesothelial cells or malignant cells in suspension *in vivo*. A small series of benign effusions from patients with an underlying malignancy (nine cases) was studied. Biochemical differences that discriminated benign from malignant effusions were identified. Significantly higher expression of AKT (P = 0.001), cAMP-responsive element binding protein (CREB; P = 0.01), c-*Jun*-NH_2-kinase (JNK; P = 0.03), p-ERK (P = 0.001), and p-CREB ($P \leq 0.001$) were observed in malignant effusions (example CREB array and analysis; Fig. 1). The extent of malignant cells did not skew this expression pattern. Partition analysis further showed that p-ERK, p-AKT, and epidermal growth factor receptors (EGFR) are discriminators of malignant from benign effusions (Supplemental Fig. S2). These biochemical events are component parts of survival and proliferation pathways. Their prominence is consistent with the expectation that malignant cells will have up-regulated autocrine survival and proliferation signaling.

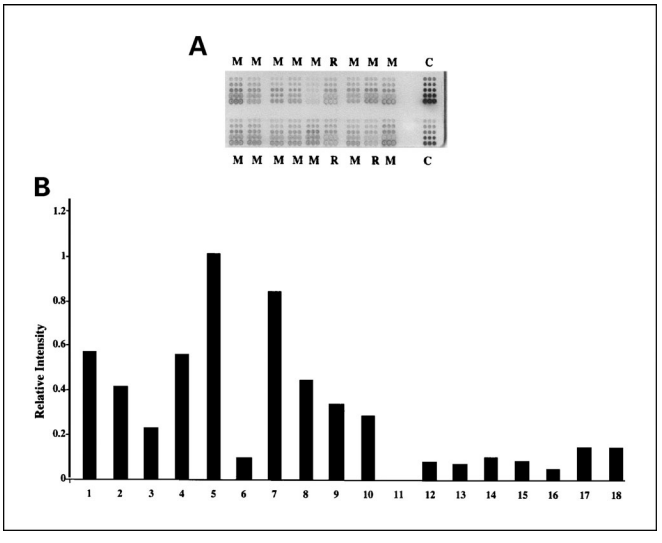

Fig. 1. Tissue lysate arrays. *A,* an example effusion lysate array of CREB expression. A series of lysates from the effusion samples were arrayed and stained for CREB expression. *B,* quantitated CREB in the samples. The array was assessed as per Materials and Methods and the results are expressed graphically.

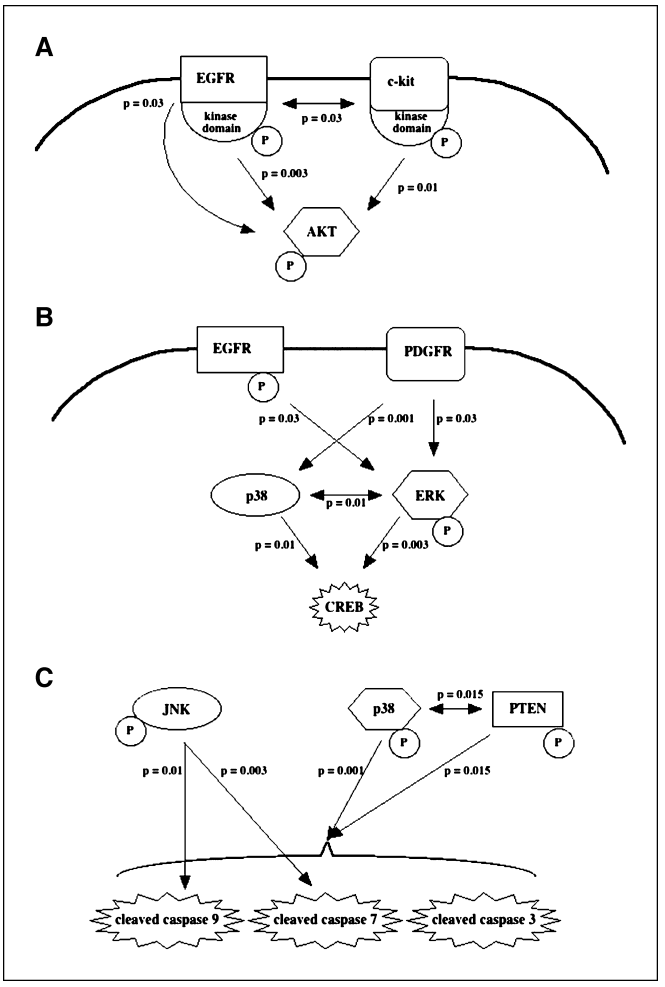

Fig. 2. Statistical analysis of signal protein expression allows organization of effusion signals into possible functional signaling pathways. P values are for paired analyses. *A,* survival pathway; *B,* proliferation pathway; *C,* apoptosis pathway.

Profiling identifies signal pathways for proliferation, survival, and apoptosis. Measured signaling events were queried to determine which biochemical events may be linked statistically to build potential pathways. Cancer cells in effusions must have up-regulated survival ability to overcome programmed cell death due to loss of matrix attachment (anoikis). Expression and biochemical activation of the proteins was analyzed to see which events were correlated and therefore might constitute linked signals in the effusions. A survival pathway was identified consisting of increased expression and activation of the receptor tyrosine kinases, EGFR and c-kit, coupled with activation of AKT (Fig. 2A). Signaling through mitogen-activated protein kinase is known to be important in proliferation and invasion (24). Analysis further correlates signals into a proliferation pathway consisting of an EGFR/platelet-derived growth factor receptor (PDGFR)-ERK/p38-CREB axis. Statistically significant expression of p-ERK

with p-EGFR and p-CREB and between PDGFR with the activated fractions of ERK and CREB is mapped in Fig. 2B. The mitogen-activated protein kinase p38 is known to be associated either with proliferative activity or apoptotic pathways. Total p38 was statistically correlated with the activation of ERK, CREB, and PDGFR in the proliferation pathway. Total and activated forms of apoptosis-related proteins were queried for their interactions in cell death pathways. PTEN is a tumor and metastasis suppressor gene, the mutation of which is associated with AKT and survival, and activation of which is associated with apoptosis (25). PTEN activation correlated in this series with expression of phospho-p38 (P = 0.015) and cleaved caspase-3 (P = 0.015; Fig. 2C). p38 phosphorylation correlated with activated (cleaved) forms of caspase-3, caspase-7, and caspase-9 (P < 0.001). Phosphorylation of JNK also correlated with cleavage of caspase-7 and caspase-9 (P = 0.01 and 0.003, respectively). These results suggest linked active pathways but do not show a functional drive of the malignant events. Dendrogram linkage revealed that the cases with the strongest expression of the apoptosis pathway proteins are specimens occurring at first recurrence (Tap2; Fig. 3, *bottom right corner*).

Differential signaling events may be predictive of or correlate with clinical outcome. Disease stage and histologic grade are

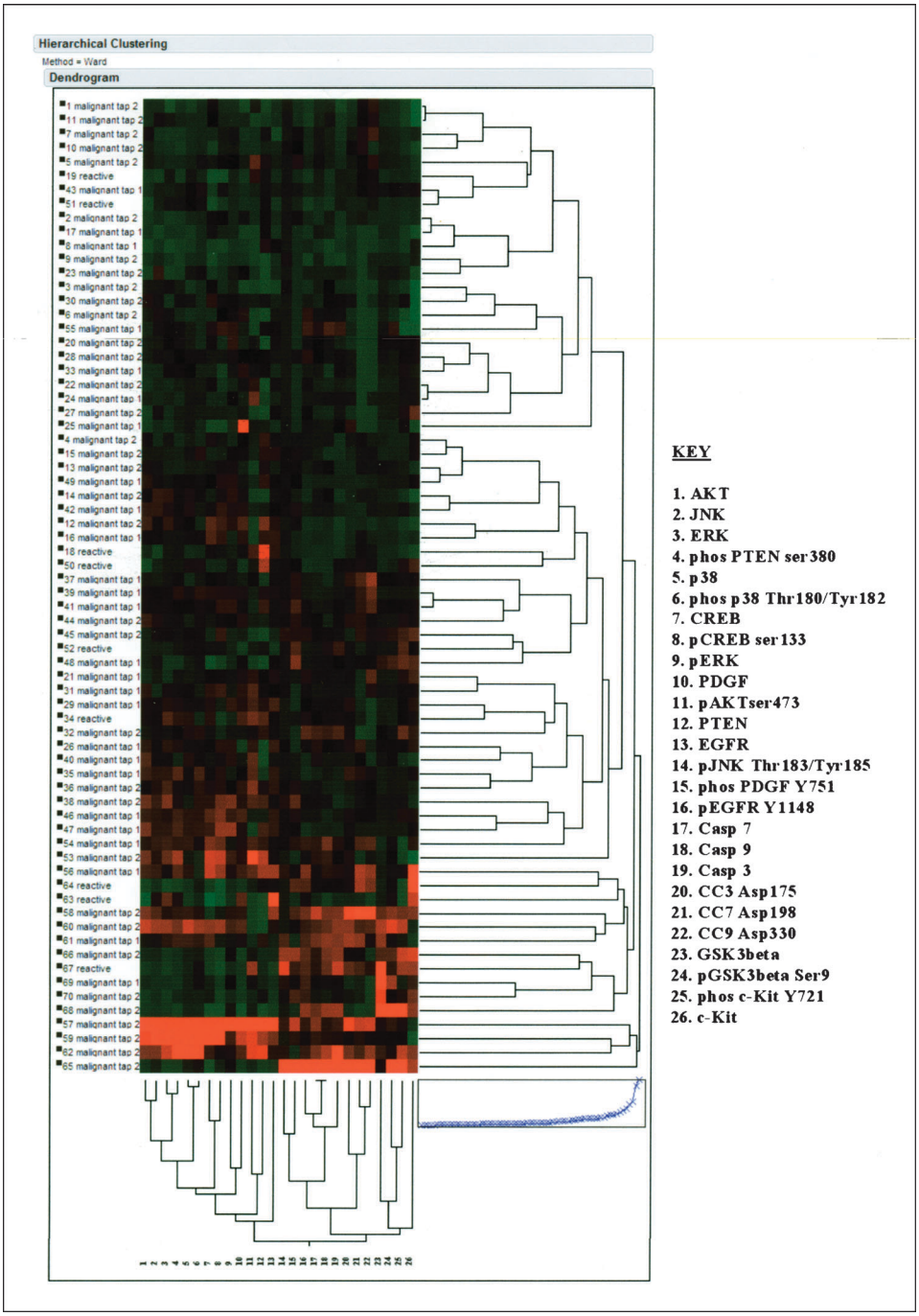

Fig. 3. Dendrogram analysis. Unbiased dendrogram analysis of expressed total and activated proteins used in all cases. The number to the left of the pathologic designation is the case tracking number and to the right, the prechemotherapy/postchemotherapy Tap1/Tap2 cohort designation.

Table 1. Univariate *P* value results from patients with Tap1 or Tap2

(A) Univariate log-rank *P* values associated with individual variables in Tap1 patients

Variable*	*P*	Variable	*P*
Disease present after diagnosis	0.019	pEGFR	0.92
Cell composition	0.36	PDGFR	0.73
Age (40-69 vs. 70+)	0.37†	pPDGFR	0.93
Ascites vs. pleural	0.61	CASP3	0.55
Grade (1 and 2 vs. 3)	0.33	CLEAVED CASP3	0.31
Residual disease (1-2 vs. 3-5 cm)	0.33	CASP7	0.066
FIGO Stage II to III vs. IV	0.11	CLEAVED CASP7 (0-1,800 vs. 1,801+)	0.085†
AKT	0.58	CASP9	0.33
pAKT 0 vs. 1+	0.39	Cleaved CASP9	0.44
ERK	0.77	AKTratio	0.95
pERK	0.26	ERK ratio	0.59
CREB	0.54	CREB ratio	0.60
pCREB	0.46	PTEN ratio	0.44
PTEN (0-900 vs. 901+)	0.044†	P38 ratio	0.16
pPTEN	0.85	JNK ratio	0.36
P38 (<1,100 vs. 1,101+)	0.01†	GSK ratio	0.60
pP38	0.07	c-KITratio	0.67
JNK	0.35	EGFR ratio (<1,525 vs. 1,526+)	0.011†
pJNK (0-267 vs. 268+)	0.085†	PDGFR ratio	0.89
GSK	0.87	CASP3 ratio	0.58
pGSK	0.39	CASP7 ratio	0.68
c-KIT	0.69	CASP9 ratio	0.39
p-c-KIT	0.47		
EGFR	0.29		

(B) Univariate log-rank *P* values associated with individual variables in Tap2 patients

Disease present after diagnosis	0.0005	PDGFR	0.74
Cell composition	0.99	pPDGFR	0.22
Age	0.90	CASP3	0.71
Ascites vs. pleural	0.11	CLEAVED CASP3	0.66
Grade (1 and 2 vs. 3)	0.31	CASP7 (<675 vs. 676+)	0.076†
Residual (1-2 vs. 3-5 cm)	0.39	CLEAVED CASP7	0.50
FIGO Stage II-III vs. IV	0.021	CASP9	0.96
AKT	0.96	CLEAVED CASP9	0.54
pAKT 0 vs. 1+	0.063	AKTratio (0 vs. 1+)†	0.069†
ERK	0.83	ERK ratio	0.18
pERK	0.75	CREB ratio	0.78
CREB	0.76	PTEN ratio (<600 vs. 601+)	0.088†
pCREB	0.53	P38 ratio	0.15
PTEN	0.76	JNK ratio	0.92
pPTEN	0.60	GSK ratio (<450 vs.451+)	0.055†
P38	0.74	CKITratio	0.27
pP38 (1-400 vs. 401+)	0.098	EGFR ratio†	0.19
JNK	0.90	PDGFR ratio	0.36
pJNK (0-325 vs. 326+)	0.0020†	CASP3 ratio	0.75
GSK	0.76	CASP7 ratio	0.61
pGSK (0-185 vs. 186+)	0.055	CASP9 ratio†	0.16
c-KIT (0-1,103 vs. 1,104+)	0.027	p-c-KIT (<820 vs. 821+)†	0.12†
EGFR	0.94	pEGFR	0.88

*Unless stated otherwise, *P* values for proteins are global results, generally comparing data from the four quartiles (see Materials and Methods).
†Does not reflect adjustment for multiple comparisons (see Materials and Methods).

Table 2. Cox model results in Tap1 and Tap2 patients

Variable	Variable estimate	P_2	HR	95% CI for HR
(A) Tap1 patients				
p38 (>1,100 vs. <1,100)	1.95	0.0023	7.01	(2.01, 24.50)
EGFR ratio (>1,525 vs. <1,525)	2.16	0.0017	8.65	(2.25, 33.46)
(B) Tap2 patients, three potential models				
Disease at diagnosis	1.45	0.0089	4.25	(1.44, 12.53)
pJNK (326+ vs. 0-325)	1.26	0.043	3.54	(1.05, 11.88)
Ascites vs. pleural	1.77	0.0093	5.87	(1.55, 22.26)
pGSK (186+ vs. 0-185)	1.77	0.0052	5.86	(1.71, 20.18)
FIGO Stage IV vs. II-III*	1.14	0.02	3.13	(1.20, 8.17)
pJNK (326+ vs. 0-325)	1.73	0.0042	5.64	(1.74, 18.28)

*One case of stage II disease.

powerful predictors of disease outcome in ovarian carcinoma (26), as in most epithelial malignancies. It is thus important to identify biochemical events that correlate with or complement these validated end points. Table 1 presents univariate *P* value results obtained from either global evaluations or evaluations based on dichotomizing the data as described in Materials and Methods. FIGO stage, PTEN, p38, pJNK, cleaved CASP7, and phospho to total EGFR ratio had $P < 0.15$ of survival for Tap1 patients, those for whom malignant effusion was present at the time of initial diagnosis (Table 1A). These were advanced into testing in a Cox model evaluation. Similarly, for Tap2 patients, those patients developing a malignant effusion at first recurrence, a relatively large number of variables with univariate $P < 0.15$ (Table 1B) were identified for inclusion in a Cox model.

Cox model analysis proceeded with these best potential features. Table 2 presents the results of the Cox models most prognostic for survival in Tap1 (A) and Tap2 (B) patients. The same results were obtained both by backward and stepwise selection. The results suggest that both elevated p38 (HR, 7.01; 95% CI, 2.01-24.50; Fig. 4A) and phospho to total EGFR ratio (HR, 8.65; 95% CI, 2.25-33.46; Fig. 4B) may be an indication of worse survival in this group of patients. Three alternative Cox models for v2 patient survival are shown in Table 2B. As an illustration, one of the three models contains the presence of ascites versus pleural fluid (HR, 5.87; 95% CI, 1.55-22.26) along with pGSK3-β (HR, 5.86; 95% CI, 1.71-20.18; Fig. 5A). Because the effusions from these patients were obtained after treatment, these results suggest that expression levels may be correlated with poor survival rather than predictive of worse outcome. Elevated pJNK levels were associated with lower survival probability in two models (Fig. 5B), coupled with the presence of disease at diagnosis and FIGO stage IV versus II/III (one case of stage II), respectively. Thus, FIGO stage, disease remaining after diagnosis, site of effusion, and quantity of pJNK and pGSK3-β, are all factors that may be considered for further investigation to explore whether results obtained from post-chemotherapy effusions may be useful in identifying patients likely to survive longer. These results suggest that lysate array proteomics may be considered as a possible tool for the analysis of biological discriminators between different prognostic patient subgroups.

Discussion

Cancer is a disease of dysregulated and aberrant protein expression and activation, stemming from genomic and transcriptional events and resulting in abnormal signaling and refractoriness to regulatory mechanisms. Recent strides have been made in uncovering important signaling pathways in ovarian cancer (9, 12, 13, 24). Much of this work was initiated from observations in patient material, but has only been validated *in vitro*. Therefore, it is important to investigate the role of the proteins and associated pathways in patient samples (27–29). Mapping of signaling pathways that mediate crucial cellular events, such as apoptosis, survival, and adhesion, provides molecular information that may become relevant for treatment decisions and prognosis (18, 28, 30). Tissue lysate protein microarrays allow assessment of the activation state, such as phosphorylation or cleavage, of a broad variety of cell signaling proteins in a high-throughput, multiplexed format that cannot be measured using other standard approaches, such as cDNA and other gene arrays (18, 30, 31). This method was applied to a series of malignant and benign pleural and peritoneal effusions to determine if a signaling signature could differentiate between benign and malignant effusions, to identify signaling pathways active in the effusions, and to discover prognostic and predictive signal events in an exploratory fashion from which further clinical investigations could be triggered. Different signaling events were identified that allow discrimination of malignant from benign effusions, and three predominant signaling pathways were identified as active in the malignant effusions. The presence of activated signaling proteins, the biochemical activity of which correlate into functional pathways, is descriptive of the malignant event. A separate analysis was done to assess signal events with potential predictive (Tap1) or correlative (Tap2) value, identifying interesting events for further study. Increased presence and activation of survival and apoptosis pathways were prevalent in the effusions sampled at first recurrence after primary chemotherapy, and could be used potentially to identify patients who had worse survival.

Metastasis of ovarian cancer cells to the pleural space is described to be due to hematogenous metastases or unexplained access from the abdominal cavity. The presence of pleural

effusion portends poor survival and is a marker of stage IV disease (2, 26, 32). One would therefore expect cancer cells in pleural effusions to possess growth and survival advantages over their counterparts in ascites. However, pathway profiling shows no biochemical distinction between pleural and peritoneal effusions to explain the differential prognostic import. The findings could be interpreted to indicate that suspended cells at either site have attained a metastatic and survival phenotype allowing them to prosper in the absence of a solid scaffolding and vascular support mechanism. Thus, the prognostic effect of pleural effusions might be related to tumor burden and local factors such as the limited potential space in the thoracic cavity and subsequent impingement on vital organs. Histologic grade, FIGO stage, and the extent of residual disease are other established predictive variables in ovarian cancer. The lack of association between signaling profiles and FIGO stage is in agreement with the lack of signal differentiation between peritoneal and pleural effusions; most stage IV patients in the study set had pleural effusion as their site of stage IV disease rather than solid metastases. An additional cohort of patients with both pleural effusion and solid tumor metastasis needs to be evaluated to further assess this question and to profile differences that may occur between solid and suspended tumor cells. Prospective case collection is ongoing to address this provocative question. Epithelial ovarian carcinoma is postulated to arise from the ovarian surface epithelium, a single cell serosal layer that resembles the benign abdominal mesothelium (33). The differential diagnosis between reactive mesothelium and ovarian carcinoma can be difficult in many cases (10). Ongoing characterization of effusions using immunocytochemistry has identified a series of molecules that are differentially expressed in malignant effusions and correlate with poor patient outcome. These include proteins in the invasion and metastasis pathways (34–37), angiogenesis (5, 8, 38, 39), and selected signaling molecules (14, 40). The current study builds on those observations by evaluating both the quantity and activation profile of a series of signaling events that define driving pathways in the effusions and cancers. As found in the earlier studies, the mitogen-activated protein kinase family, especially ERK, is prominent in the malignant effusions, and was key in one of the discriminatory arms identifying reactive from malignant effusions (Fig. 2). The higher expression of activated ERK, AKT, and CREB, in combination with total AKT, discriminated malignant effusions and poor survival outcomes. This is consistent with what is known about the function of these

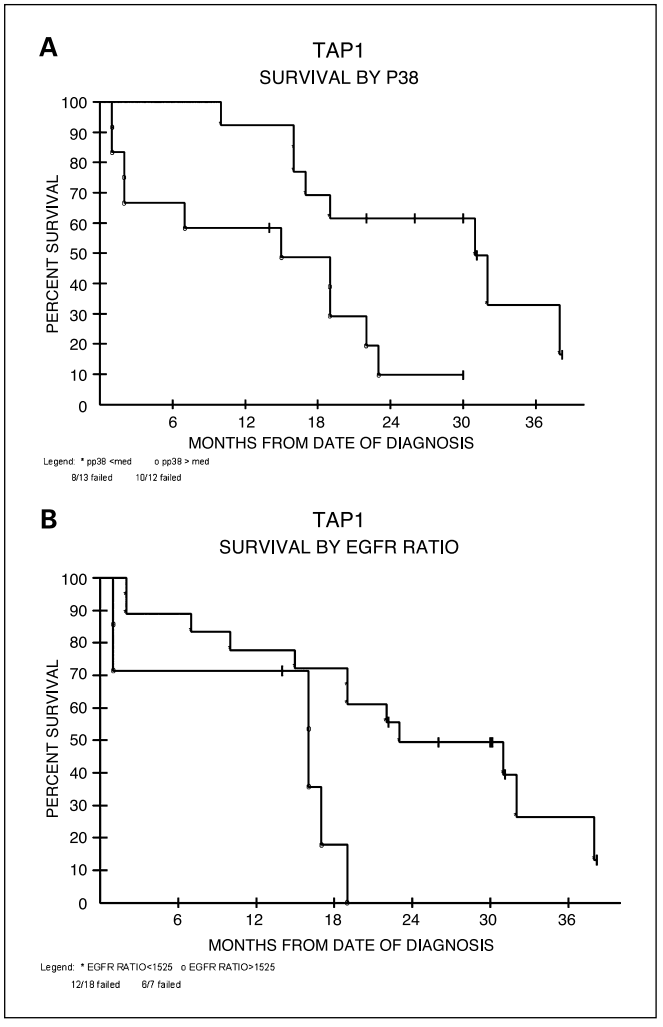

Fig. 4. Survival curves. Kaplan-Meier survival probability analysis for Tap1 patients by p38 (*A*) and EGFR ratio (*B*).

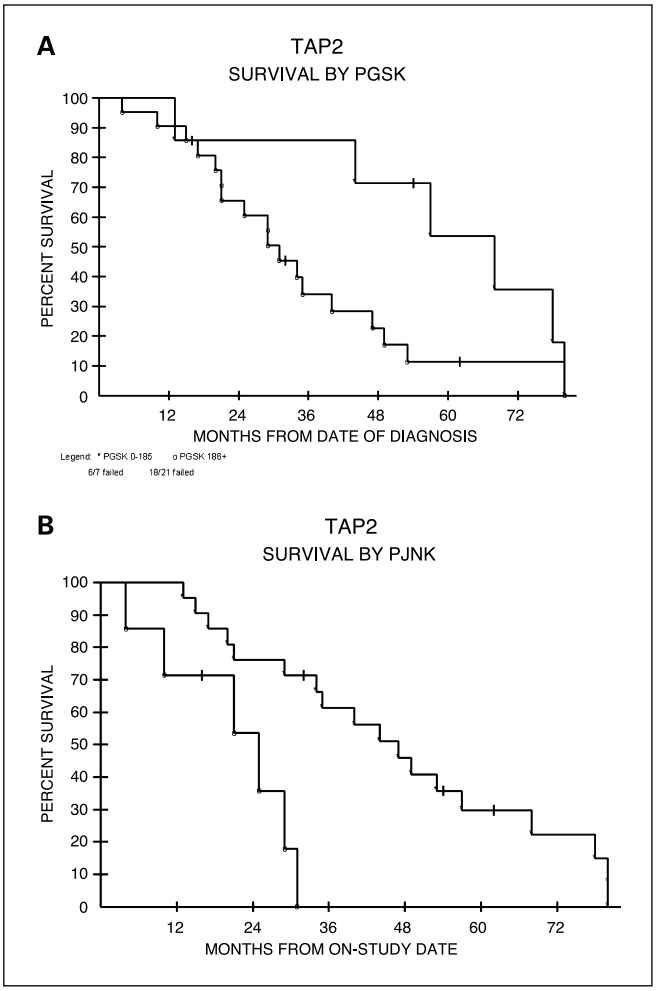

Fig. 5. Survival curves. Kaplan-Meier survival probability analysis for Tap2 patients by phospho-GSKβ (*A*) and phospho-JNK (*B*).

pathways in proliferation and survival, favoring both growth and reduced apoptosis. AKT is the canonical survival protein (15). Its presence correlated with malignancy over benign effusions and was also important in segregating those patients with poor survival outcome, as would be expected for up-regulation of malignant cell survival events. Signaling through CREB is known to stimulate the production of other growth factors, supporting autocrine loops (41). The proteomic signal profiling of these effusions further supported the identification of mitogen-activated protein kinase and AKT as molecular targets in ovarian cancer and builds on their potential utility by showing prognostic load in the effusions.

Defining signaling pathways in cancer cells through proteomic analysis offers the possibility of designing drugs and optimizing use of molecularly targeted agents against biologically active and important pathways. Analyses of coexpression in our cohort suggests that activation of signaling via AKT and ERK is by tyrosine kinases such as EGFR and PDGFR, with nuclear activation of CREB and other transcription factors. Inhibitors of these receptors (42–44) and those signaling pathways (45) are currently the target of extensive clinical trials in cancer. These data may aid in choosing patients for treatment whose tumor cells show increased activation of these targeted pathways. Similarly, identifying patients who respond to treatment by showing JNK activation and caspase cleavage, as seen in a subset of our patients, may aid in identifying patients that respond favorably to chemotherapy.

The exploratory univariate analyses and subsequent Cox models presented in this analysis suggest that data on protein levels from effusions taken at the time of diagnosis or after chemotherapy may be of some value in prediction of or be a correlate of survival. The cut-points presented in this report should be interpreted as approximate and suggestive but may serve as tools for further hypothesis generation. The findings could be considered limited by the number of patients studied, the limited number of antibodies being assessed, and, although enriched for malignant cells (>80%), the differing percentages of malignant cells. This is proposed as a starting point and suggests that large studies evaluating several of the variables explored in this study should be given consideration.

The clinical outcome of the Tap1 and Tap2 patients were different. This may be a result of the fact that clinical presentation with pleural effusion (stage IV) and ascites (all stages) are poor prognostic factors, and are rare in the patients who have long-term disease-free survival, within their respective stages (46). Progression or recurrence of disease defines incurable disease independently of the presence of effusions. Our two groups of patients, Tap1 patients, those for whom the effusions were present prior to initial therapeutic intervention, and Tap2 patients, for whom the effusions developed and were sampled at first disease recurrence, had different median disease-free intervals. Clinical variables were obtained on patients in both groups, allowing the assessment of biochemical discriminators in each group individually. This difference in median disease-free survival was observed despite the comparable clinicopathologic profiles and the similar treatment programs for the two groups who were ascertained simultaneously. A comparative analysis of expression and activation of the studied molecules showed significant differences only in total p38 expression, raising the possibility that additional untested molecules are involved in these pathways. Survival analyses done separately showed that different pathways may be prominent in these two clinical groups. Increased expression of c-kit and activated AKT in Tap2 patients suggests that tumor cells that maintain pro-survival signaling following chemotherapy are viable and associated with worse survival. This is also in agreement with *in vitro* studies linking inhibition of the type III tyrosine kinase receptors, c-kit, PDGFR, and abl, by imatinib mesylate, with inactivation of AKT (43, 47), and the role of AKT in negating the response to chemotherapy (48, 49). These data support the need to continue and expand proteomic analysis of total and activated signaling molecules in ovarian cancer effusions and solid tumors. Lysate array proteomics is a new tool with which to characterize the biochemical pathways underlying benign and malignant effusions and from which to profile biochemical events in ovarian and other cancers. Mapping survival- and apoptosis-related events provides data from which to prioritize targeted therapy development and advances one of the critical building blocks leading to improved molecular therapeutics and personalized molecular medicine.

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